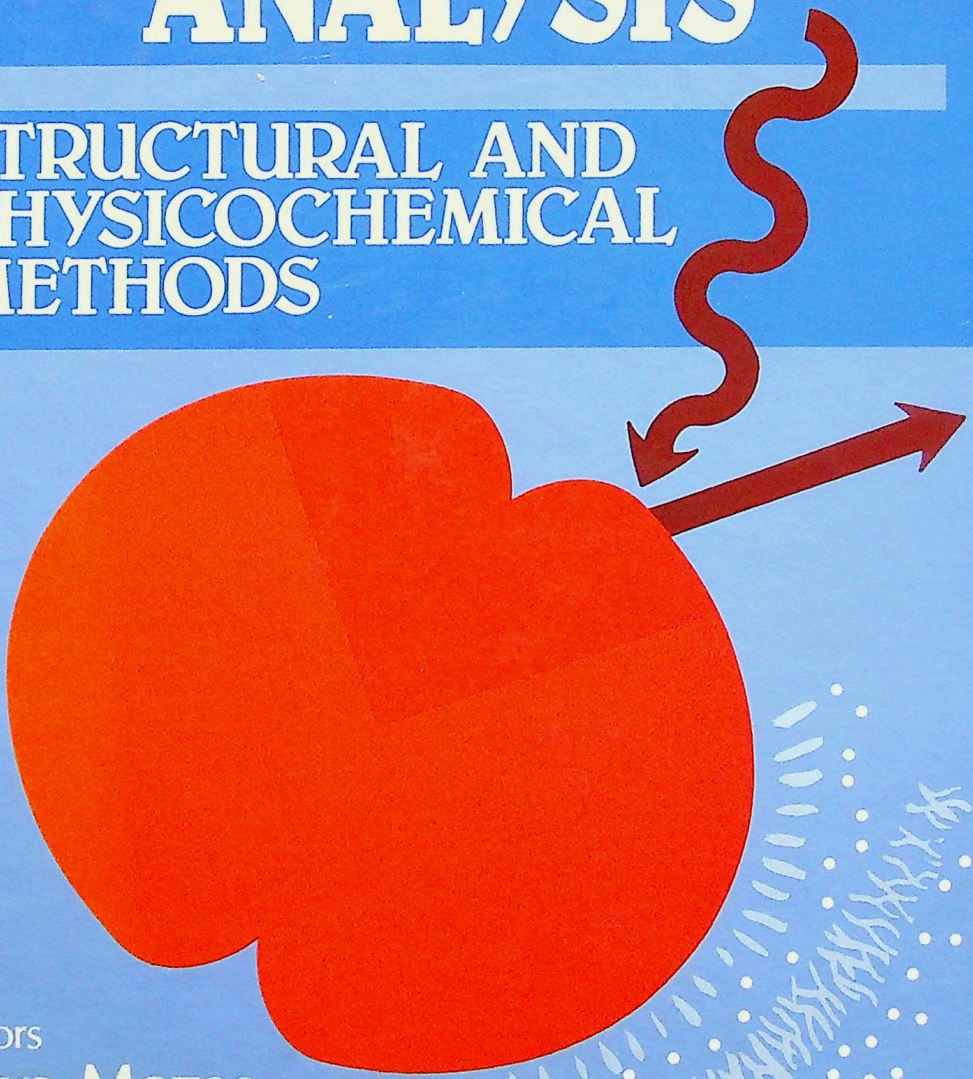
MICROBIAL CELL SURFACE ANALYSIS

STRUCTURAL AND PHYSICOCHEMICAL METHODS



Editors
Nava Mozes
Pauline S. Handley
Henk J. Busscher
Paul G. Rouxhet

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Microbial Cell Surface Analysis

Structural and Physicochemical Methods

Edited by

Nava Mozes
Pauline S. Handley
Henk J. Busscher
Paul G. Rouxhet

Nava Mozes
Laboratory of Chemistry of Interfaces
Catholic University of Louvain
Place Croix du Sud 1
B-1348 Louvain-la-Neuve
Belgium

Pauline S. Handley
Department of Cell and Structural Biology
University of Manchester
Stopford Building
Oxford Road
Manchester M13 9PT
United Kingdom

Henk J. Busscher
Laboratory for Materia Technica
University of Groningen
Antonius Deusinglaan 1
9713 AV Groningen
The Netherlands

Paul G. Rouxhet
Laboratory of Chemistry of Interfaces
Catholic University of Louvain
Place Croix du Sud 1
B-1348 Louvain-la-Neuve
Belgium

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United Kingdom

Contributors

Julian E. Beesley, Department of Pharmacology, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, United Kingdom

Michael R.W. Brown, Pharmaceutics Research Institute, Aston University, Aston Triangle, Birmingham B4 7ET, United Kingdom

Henk J. Busscher, Laboratory for Materia Technica, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

Gordon Dougan, Department of Molecular Biology, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, United Kingdom

Ron J. Doyle, Academic Health Center, University of Louisville, Louisville, KY 40292, U.S.A.

Ian G. Duguid, Department of Pharmacy, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom

David J. Evans, Department of Pharmacy, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom

Elwyn Evans, Department of Pharmacy, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom

Michel J. Genet, Laboratory of Chemistry of Interfaces, Catholic University of Louvain, Place Croix du Sud 1, B-1348 Louvain-la-Neuve, Belgium

Peter Gilbert, Department of Pharmacy, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom

Ian C. Hancock, Department of Microbiology, Newcastle University, The Medical School, Framlington Place, Newcastle-upon-Tyne, United Kingdom

Pauline S. Handley, Department of Cell and Structural Biology, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, United Kingdom

Louis M. Hesketh, Department of Cell and Structural Biology, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, United Kingdom

Jan A. Hobot, Electron Microscope Unit, University of Wales, College of Medicine, Heath Park, Cardiff CF4 4XN, United Kingdom

Arthur M. James, 50, Brodrick Road, Tooting, London SW17 7DY, United Kingdom

Kevin C. Marshall, School of Microbiology, The University of New South Wales, P.O. Box 1, Kensington, New South Wales 2033, Australia

Paul Messner, Center for Ultrastructure Research, University of Agriculture, Gregor Mendel Street 33, A-1180 Vienna, Austria

Mel Rosenberg, Laboratory of Oral Microbiology, Tel-Aviv University, Maurice and Gabriela Goldschleger School of Dental Medicine, Sackler Faculty of Medicine, Ramat-Aviv 69978, Israel

Paul G. Rouxhet, Laboratory of Chemistry of Interfaces, Catholic University of Louvain, Place Croix du Sud 1, B-1348 Louvain-la-Neuve, Belgium

Uwe B. Sleytr, Center for Ultrastructure Research, University of Agriculture, Gregor Mendel Street 33, A-1180 Vienna, Austria

Henny C. van der Mei, Laboratory for Materia Technica, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

Preface

The surface of the microbial cell is of paramount importance for cell survival, as it controls the processes of association between the cell and other surfaces, such as adhesion, biofilm formation, flocculation, formation of aggregates, and flotation. The cell surface also triggers the host immune response. Such cell surface-related phenomena play a crucial role in a number of disciplines, including medicine, environmental protection, biotechnology, material technology, and process engineering.

Biologists and physical chemists need to collaborate closely in trying to elucidate the fundamental processes involved in cell surface interactions with the environment. Biologists are concerned with the highly complex, ever changing nature of the cell surface, features that are sometimes ignored by physical chemists. The latter generally envisage the bacterial cell in terms of model colloids, which in turn are not familiar to biologists. The aim of this book is, therefore, to bridge the gap of understanding that exists between these two disciplines.

The book presents the methods currently used to investigate microbial cell surfaces. Some of the techniques are well established whereas others have been developed only recently. The basic principles, practical aspects, and applications of the techniques are described and data interpretation is discussed. A critical analysis of the techniques is also included, as well as an assessment of their future potential.

The terms cell wall and cell surface are often used confusingly. In this book the cell surface is regarded as the zone that is in direct contact with the external environment; the exact location of this zone depends on the technique used to study it. In the introductory part of the book the importance of studying microbial cell surfaces is emphasized and a thorough description of our present knowledge of the architecture of the entire cell wall and cell surface layers is presented. The second part is devoted to the characterization of the structure of microbial cell surfaces by electron microscopic techniques including negative staining, carbohydrate detection, freeze-etching, freeze-drying, freeze-substitution, low-temperature embedding, and immunolabeling. The third part covers methods that provide information on the atomic and molecular composition of cell surfaces and on their physicochemical properties such as electrical charge, hydrophobicity, and surface energy. The last part contains specific studies

in which both physicochemical and structural methods, described in the book, are integrated to investigate the cell surface in relation to adhesion.

This book is multidisciplinary in its approach and is intended to help novice and experienced researchers, engineers, and physical chemists as well as biotechnologists and microbiologists. We hope that by addressing such a large audience it will help to generate ideas and collaborative studies of the future.

We would like to express our deep gratitude to the authors for their commitment to the task. It was not easy to compromise between the fundamentally simple yet highly rigorous approach necessary to generate a book comprehensible at all levels.

Nava Mozes
Pauline S. Handley
Henk J. Busscher
Paul G. Rouxhet

10

Assessment of Microbial Cell Surface Hydrophobicity

Henny C. van der Mei
Mel Rosenberg
Henk J. Busscher

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10.1. INTRODUCTION

Bacterial hydrophobicity is a term used to describe the hydrophobic properties conferred on bacterial cells by their outermost cell surfaces.

In physical chemistry, the implications of the words "hydrophobic" or "hydrophilic" seldom go beyond the macroscopic wetting behavior of a surface by water in air (Hoffman, 1986). Thus teflon and paraffin wax are called hydrophobic because a water droplet does not spread, and clean glass is called hydrophilic for the opposite reason. Hydrophobicity is not necessarily associated with repulsion between water and a surface (Tanford, 1979). Even between water and hydrocarbons attractive interactions exist, which are however smaller than the attraction of water for itself. This relative lack of attraction between water and a surface forms the basis for the term "hydrophobicity" (Tanford, 1979).

The task of describing the hydrophobicity of microbial cell surfaces is daunting and much more complicated than in the case of solid surfaces. There is no universal definition of "bacterial hydrophobicity" and generally the distinction between more or less hydrophobic strains has been made on the basis of

low or high water contact angles on bacterial lawns (Absolom *et al.*, 1983; Busscher *et al.*, 1984; Van Pelt *et al.*, 1984; Van Loosdrecht *et al.*, 1987);

low or high adsorption to hydrocarbon droplets in a bacterial suspension (Gibbons and Etherden, 1983; Rosenberg *et al.*, 1980; Lichtenberg *et al.*, 1985; Sweet *et al.*, 1987; Vanhaecke and Pijck, 1988);

low or high adsorption to octyl- or phenyl-sepharose in hydrophobic interaction chromatography (Jonsson and Wadström, 1983; Stenström, 1989);

high or low amounts of salting-out agents required to induce aggregation of a bacterial suspension (Lindahl *et al.*, 1981; Ljungh *et al.*, 1985);

low or high affinity for polyethylene glycol in two-phase partitioning (Miörner *et al.*, 1982; Malmqvist, 1983); and

low or high adsorption of nonionic (Noda and Kanemasa, 1986) or anionic surfactants (Dyar, 1948; Hill *et al.*, 1963).

The enormous rise in scientific interest regarding bacterial hydrophobicity (Rosenberg and Kjelleberg, 1986) is due to its ubiquitously accepted importance in microbial adhesion. Busscher and Weerkamp (1987) proposed that the main role of bacterial hydrophobicity in adhesion is to remove the water film between interacting surfaces, thereby enabling specific short-range interactions to occur.

Positive relations between bacterial hydrophobicity and their adhesion to solid substrata have been found in dental research (Pratt-Terpstra *et al.*, 1988), marine fouling (Fletcher and Loeb, 1976), adhesion of fresh water bacteria (McEldowney and Fletcher, 1986), microbial adhesion to minerals (Stenström, 1989), and medical implants and host colonization related diseases (Hogt *et al.*, 1982).

In this chapter, several experimental methods to determine bacterial hydrophobicities will be reviewed critically and results of various tests will be compared.

10.2. CONTACT ANGLE MEASUREMENTS AND ESTIMATION OF SURFACE FREE ENERGIES

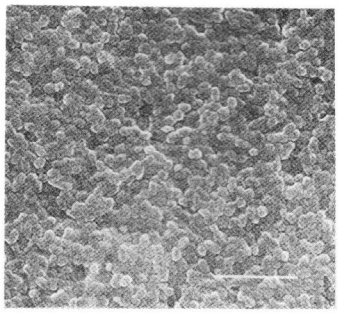
10.2.1. Contact Angle Measurements

Contact angle measurements are often used to obtain an indication of the hydrophobicity of solid surfaces. The interpretation of contact angle data is easiest for ideally smooth and homogeneous surfaces. Deviations from the ideal state may give rise to so-called contact angle hysteresis, a difference between advancing and receding contact angles (Johnson *et al.*, 1977).

Obviously, ideal conditions can never be met for bacterial cell surfaces. Usually it is advocated that a uniform layer of cells is produced on agar (Van Oss *et al.*, 1975) or that bacteria are deposited on membrane filters (Van Oss *et al.*, 1975; Busscher *et al.*, 1984; Van der Mei *et al.*, 1987b) in order to produce as uniform a surface as possible.

We prefer the latter (membrane) technique, as cells are less apt to detach into the liquid phase and there is generally less influence on the contact angle of the membrane surface properties than of the underlying agar. The best way to reproducibly produce bacterial lawns on membrane filters is to slowly deposit approximately 50 layers of bacteria, suspended in water, on a membrane filter by applying negative pressure (Figure 10-1). These wet membranes with bacterial preparations can then be attached on sample holder plates with, for example, double sided adhesive tape and dried under standardized conditions. To determine whether the cells are homogeneously deposited, scanning electron micrographs can be made periodically (Figure 10-1).

A variety of experimental methods to measure contact angles have been described in the literature (Neumann and Good, 1979). The basic requirements



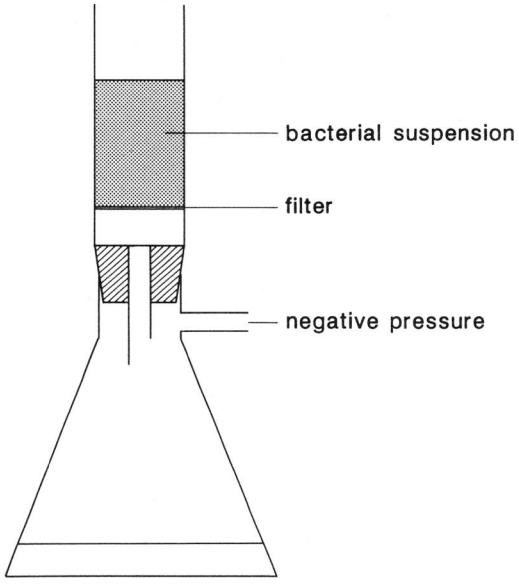


Figure 10-1 Experimental setup for the preparation of bacterial lawns on membrane filters and a scanning electron micrograph of a bacterial lawn thus produced (*Streptococcus mutans* NS). The bar denotes 10 μm.

consist of a solid support system, a microsyringe for positioning the droplets, and viewing optics with suitable measuring equipment. In a very simple setup, the contact angles are measured with a goniometer, a slow and highly observer-dependent method. Alternatively, if the droplets have a spherical shape, the contact angle θ can be inferred from the measured height, h, and base width, b, of a droplet according to

$$\theta = 2 \tan^{-1} \frac{2h}{b} \qquad (10\text{-}1)$$

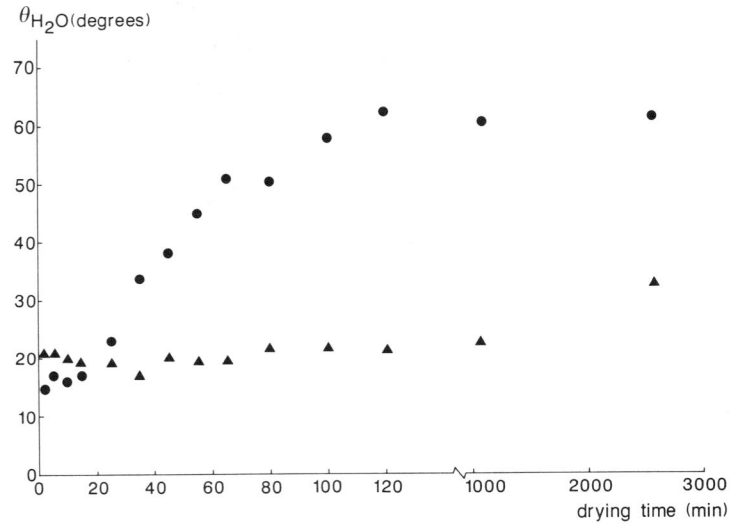


Figure 10-2 Water contact angles θ_{H_2O} on a lawn of *Serratia marcescens* RZ (●) and *Serratia marcescens* 3164 (▲) cells as a function of the drying time. Note that a drying time of at least 100 min is required for the hydrophobic strain RZ under these experimental conditions before a plateau is reached. After excessively long drying the contact angle on the hydrophilic strain (3164) starts to increase again.

This method is also slow, but much more observer independent. We use an automated, real-time image analysis system, specially constructed for contact angle measurements on sessile drops (Electronisch Ontwerpbureau de Boer, Groningen, the Netherlands), based on black and white thresholding techniques. This more modern technique is fast (approximately one contact angle measurement per second) and observer independent.

Prior to measuring definitive contact angles on these bacterial preparations, the evaporation of water has to be monitored by measuring the water contact angle as a function of the drying time (Busscher *et al.*, 1984), particularly when starting out with new strains. Generally, a plateau in the drying time curves can be observed after 1–4 hr drying, dependent on the strains and conditions employed. These plateau contact angles are considered throughout as the physiologically relevant ones (Van Oss *et al.*, 1975). Figure 10-2 shows an example of such a drying time curve. Excessive drying should be avoided as this may lead to the collapse of hydrophobic structures on the cell surface.

Sometimes contact angles change as the drop spreads are reported to be troublesome (Sar, 1987) and a strong time dependence of the contact angles after droplet positioning is observed. Often this is due to stacking too small a number of cells on a filter or to insufficient evaporation of water from the bacterial film. Originally we proposed to extrapolate the measured contact angles to $t = 0$ in order to account for these stability problems. Using real-time analysis of the time

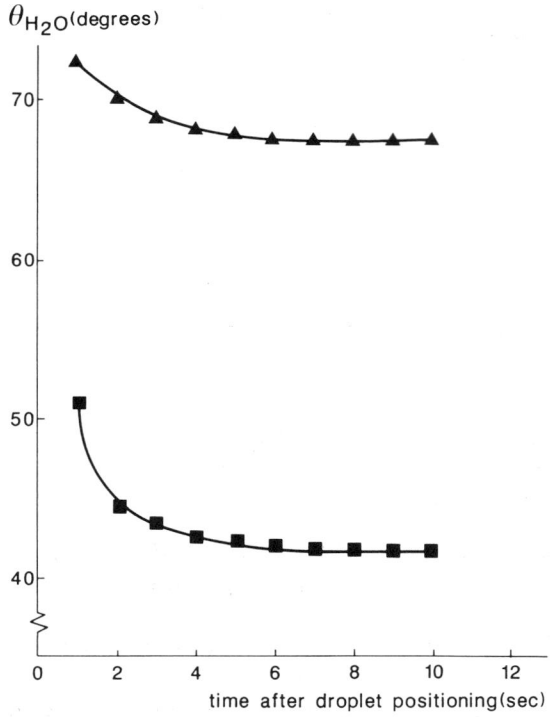


Figure 10-3 Contact angle θ_{H_2O} of a water droplet on polymethylmethacrylate (▲) and on a lawn of *Serratia marcescens* RZ30 (■) cells as a function of the time after positioning the droplet. Note that the establishment of an equilibrium takes 5–7 sec on bacterial lawns as well as on inert polymers.

dependence of contact angles we found, however, that even on polymers the establishment of a contact angle equilibrium takes 5–7 sec; hence it is probably better to measure the contact angles on bacterial lawns also 5–7 sec following application of the droplet rather than extrapolating back to $t = 0$ (Figure 10-3).

Table 10-1 gives contact angles measured with pure liquids on a variety of bacterial strains.

10.2.2. Estimation of Surface Free Energies

Although the measurement of contact angles on bacterial lawns may be called controversial from an experimental point of view, the estimation of surface free energies from observed contact angles is controversial from a theoretical point of view as well (Sacher, 1983). Several, often controversial, thermodynamic schemes have been forwarded to derive surface free energies from contact angles. A complete treatise of all approaches described is beyond the scope of this chapter.

Table 10-1 A Collection of Contact Angles of Different Liquids (Degrees) Measured on Lawns of Various Bacterial Strains

Strain	Water	Formamide	Diiodomethane	α-Bromo-naphthalene	Hexadecane
Streptococcus mitis T9	91	n.d.[a]	n.d.	36	n.d.
Streptococcus mutans NS	26	n.d.	n.d.	37	<10
Serratia marcescens RZ	52	30	58	47	5
Serratia marcescens 3164	24	25	59	49	5
Leuconostoc mesenteroides NCDO523	17	39	52	36	n.d.

[a]n.d., not determined.

It is useful, however, to illustrate that large differences can arise in surface free energies derived according to different theoretical approaches. To this end, we will briefly discuss and compare results of four approaches:

1. the Neumann equation of state (Neumann *et al.*, 1974);
2. the geometric-mean equation neglecting spreading pressures (Owens and Wendt, 1969);
3. the geometric-mean equation accounting for spreading pressures (Busscher *et al.*, 1983); and
4. the Lifshitz–van der Waals/acid–base approach (Van Oss *et al.*, 1987, 1988).

Table 10-2 lists the surface free energies estimated for the strains in Table 10-1 according to the above four approaches.

For completeness, it should be added that the empirical critical surface tension concept, as originally developed by Zisman and co-workers (1963, 1964), is still used by some groups studying bioadhesion (Baier *et al.*, 1985; Dexter *et al.*, 1975). It is based on characterizing a surface by the surface tension of a hypothetical liquid that completely wets that surface. The surface tension of this hypothetical liquid is derived by linear extrapolation of the cosines of measured contact angles versus the liquid surface tensions in order to find the surface tension at which the contact angle is zero (i.e., $\cos \theta = 1$). Although the concept is simple and not under attack from theoreticians, as it is a purely empirical approach, application is difficult. Often linear extrapolation yields very low coefficients of correlation and one is forced to discard a major part of the data points involved. For these reasons, most researchers prefer the above listed, less empirical approaches and we have decided not to discuss further the critical surface tension concept.

The Neumann equation of state is based on the assumed existence of one equation, relating the three interfacial surface free energies involved in the contact angle equilibrium (Figure 10-4) described by Young as

$$\gamma_{lv} \cos \theta = \gamma_{sv} - \gamma_{sl} \qquad (10\text{-}2)$$

Table 10-2 Surface Free Energies of Various Microbial Strains (From Table 10-1) According to Different Thermodynamic Approaches to Convert Measured Contact Angles into Surface Free Energies[a]

Strain	*Streptococcus mitis* T9	*Streptococcus mutans* NS	*Serratia marcescens* RZ	*Serratia marcescens* 3164	*Leuconostoc mesenteroides* NCDO523
Neumann Equation of State[b]					
γ_{sv}, water	28.5	66.4	52.2	67.2	70.0
γ_{sv}, α-bromonaphthalene	36.4	36.0	31.9	31.1	36.4
Geometric-Mean Equation Neglecting Spreading Pressures[c]					
γ_{sv}^{d}	—[f]	–	23.4	20.8	30.2
γ_{sv}^{p}	–	–	20.6	33.6	35.3
γ_{sv}	–	–	44.0	54.5	65.5
Geometric-Mean Equation Accounting for Spreading Pressures[d]					
γ_{s}^{d}	36.3	35.9	31.4	30.4	36.2
γ_{s}^{p}	2.7	81.1	51.5	90.5	83.2
γ_{s}	39	117.0	82.9	121.0	119.4
Lifshitz–van der Waals/Acid–Base[e]					
γ_{sv}^{LW}	–	36.1	30.6	29.8	34.8
γ_{sv}^{-}	–	51.5	21.6	51.9	68.3
γ_{sv}^{+}	–	0.8	3.7	2.4	0.2
γ_{sv}^{AB}	–	13.3	17.7	22.5	7.4
γ_{sv}	–	49.3	48.3	52.3	42.2

[a]All surface free energies in this table are expressed in SI units: mJ m^{-2} (the equivalent unit in the CGS-system is erg cm^{-2}).
[b]Using the equation of state approach, contact angles of both water and α-bromonaphthalene were employed to yield two surface free energy values (Neumann *et al.*, 1974).
[c]In this approach, the contact angles of various pure liquids (see the contact angle data in Table 10-1) are employed (Owens and Wendt, 1969; Kaelble, 1970).
[d]This method requires angle measurements with water, water/*n*-propanol mixtures (not presented), and α-bromonaphthalene (Busscher *et al.*, 1983).
[e]This approach has been included, using the contact angles of water, formamide, diiodomethane, and α-bromonaphthalene (Van Oss *et al.*, 1987, 1988).
[f]Limited number of liquids with known contact angles for this strain inhibits the calculation of surface free energies according to this approach.

This implies that surface free energies can be calculated once the contact angle of one liquid of known surface free energy is measured. To this end computer programs and tables have been made available (Neumann *et al.*, 1980). Results for the strains listed in Table 10-1, obtained with published computer tables, using both the water and α-bromonaphthalene contact angles, are shown in Table 10-2. This table demonstrates a severe drawback of the equation of state: the surface free energies derived depend very strongly on the type of liquid involved. This makes

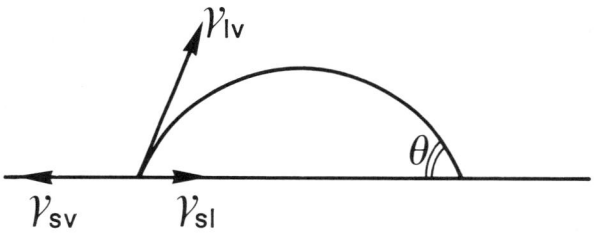


Figure 10-4 The contact angle equilibrium. γ_{ij} denotes the interfacial free energy between solid (s), liquid (l), and vapor (v); θ is the contact angle. The Young equation, Eq. (10-2), which is basic to all thermodynamic schemes to determine surface free energies from measured contact angles, can be inferred from a simple force balance parallel to the surface.

a proper interpretation of surface free energies thus derived impossible, unless the type of liquid employed and the exact contact angle data are specified.

The geometric-mean equation is based on the assumption that surface free energies can be separated into a dispersion γ^d and polar γ^p component, which can be combined according to a geometric-mean, i.e.,

$$\gamma_{12} = \gamma_1 + \gamma_2 - 2(\gamma_1^d \gamma_2^d)^{1/2} - 2(\gamma_1^p \gamma_2^p)^{1/2} \qquad (10\text{-}3)$$

Several calculational schemes are available in the literature (Owens and Wendt, 1969; Dann, 1970a,b; Kaelble, 1970; Busscher *et al.*, 1983) to allow determination of surface free energies from a combination of Eq. (10-3) with the Young equation, Eq. (10-2), employing contact angle data of several liquids with known surface free energy components. The data presented in Table 10-2 show that the total surface free energy derived from this approach gives results very much in line with those based on the equation of state using water contact angles, but not α-bromonaphthalene contact angles; α-bromonaphthalene-based surface free energies from the equation of state seem more closely related to the dispersion components.

Both above-mentioned approaches neglect the effect of the so-called spreading pressure. Spreading pressure is the reduction in surface free energy of a material due to adsorption of vapor molecules. The three phase boundary region at the edge of a droplet constitutes a region of saturated vapor. Hence the surface free energy probed by a liquid droplet is not always the real surface free energy γ_s but expresses a reduced value γ_{sv}. The spreading pressure π_e is defined as

$$\pi_e = \gamma_s - \gamma_{sv} \qquad (10\text{-}4)$$

It is not unequivocally accepted that spreading pressures may be neglected (Hu and Adamson, 1977; Busscher *et al.*, 1986), although it is usually done. Those approaches that do account for spreading pressures yield elevated surface free energies, as compared to those which do not (Table 10-2). Note that data accounting

for spreading pressure are referred to as γ_s data; when spreading pressures are neglected the subscript γ_{sv} is used.

The final approach discussed has recently been developed by Van Oss *et al.* (1987, 1988) and is based on an even more extensive separation of surface free energies in a Lifshitz–van der Waals γ^{LW} component, which is more or less similar to the dispersion component and an acid–base γ^{AB} component. The acid–base component is said to describe hydrogen bonding. Accordingly γ^{AB} can be separated in a hydrogen-accepting γ^- and hydrogen-donating γ^+ surface free energy parameter:

$$\gamma^{AB} = 2(\gamma^- \cdot \gamma^+)^{1/2} \qquad (10\text{-}5)$$

This approach yields the most detailed surface energetic characterization of a material, provided liquids with known characteristics are employed (Table 10-2).

Unfortunately, no decisive conclusion can be given at present on which approach is best. All four methods discussed have a scientific basis, however, and are currently employed for the characterization of microbial cell surfaces. Therefore interpretation of surface free energies should be done with great caution and it is thus advisable to pay more attention to the contact angle data than to the surface free energies. Therefore we suggest that researchers publish their contact angles together with the surface free energy derived according to the approach of their choice.

Finally we note that bacterial hydrophobicities assessed by means of contact angles with liquids on bacterial lawns can be used in thermodynamic approaches (Absolom *et al.*, 1983; Busscher *et al.*, 1984) predicting bacterial adhesion to substrata, as bacterial cell surface free energies can be estimated from measured contact angles (Owens and Wendt, 1969; Neumann *et al.*, 1974; Busscher *et al.*, 1983; Van Oss *et al.*, 1987). According to Bellon-Fontaine *et al.* (1990) only the approach based on polar and dispersion components while accounting for spreading pressures correctly predicts experimentally observed trends in the adhesion of microorganisms to solid substrata. This may be one of the reasons researchers prefer this approach, although, as stated before, one should remain very critical when deriving surface free energies from measured contact angles.

10.2.3. Purity Control of the Liquids

To obtain the best results, it is strongly recommended that the surface tension of the liquids used is regularly checked and compared with published literature values (Weast, 1979). This recommendation is particularly important for contact angle measurements, but probably also for the BATH test and HIC.

Pure liquids contaminate very easily. The purity of water decreases rapidly when stored under normal atmospheric conditions and tap water or even distilled or demineralized water is not necessarily adequate for contact angle measurements. We have had the best results with freshly tapped, Millipore Q water for

atomic absorption analysis. Other liquids, for example, diiodomethane or α-bromonaphthalene, are prone to spontaneous oxidation in air, also affecting their surface tensions.

Various, more or less sophisticated instruments are commercially available at present for liquid surface tension measurements. Simple instruments consist of a force balance and a ring or plate of a highly wettable material (e.g., platinum) and with a well-defined circumference. The ring or plate is then pulled out of a liquid reservoir and the maximum force required to rupture the liquid film is recorded. In more sophisticated instruments all procedures are microprocessor controlled and automatically registered.

The surface tensions are subsequently calculated from the measured maximum force while accounting for the weight of the liquid film (for details see Adamson, 1976). Although surface tension measurements do not yield an absolute guarantee for the purity of liquids, it usually is a sufficient control with respect to those experiments, relying on interfacial phenomena.

10.3. ADHESION TO HYDROCARBONS

10.3.1. Original BATH Test

Bacterial adhesion to hydrocarbons (BATH) is a simple method for a rapid screening of the relative hydrophobicity of bacterial strains (Rosenberg *et al.*, 1980). However, details of the actual experimental setup may greatly affect the results.

The method is based on the partitioning of cells having hydrophobic surface characteristics at the interface of a biphasic hydrocarbon–aqueous system after brief mixing.

The BATH test, as originally described by Rosenberg *et al.* (1980) can be summarized as follows: a turbid, aqueous suspension of washed microbial cells is mixed by vortexing in the presence of a test liquid hydrocarbon under well-controlled conditions in a glass test tube. The volumes and ratios of the phases employed and also the bacterial concentration should be chosen in such a way that the hydrocarbon phase is not "overloaded." Since it is impossible to suggest one appropriate phase ratio, it is advised to use different volumes of hydrocarbon with one bacterial concentration, so that "overloading" of the hydrocarbon phase can be effectively avoided. Following mixing, the two phases are allowed to separate. In the case of a hydrophobic strain, cells from the aqueous phase become bound to hydrocarbon droplets and rise with these droplets after mixing to form an upper "cream" consisting of cell-coated oil droplets (Figure 10-5). When hydrophilic nonadherent bacteria are tested, the phases separate following the mixing procedure, but the cells remain in the bulk, aqueous phase. The percentage of cells adhering to the hydrocarbon phase is considered to be a measure of

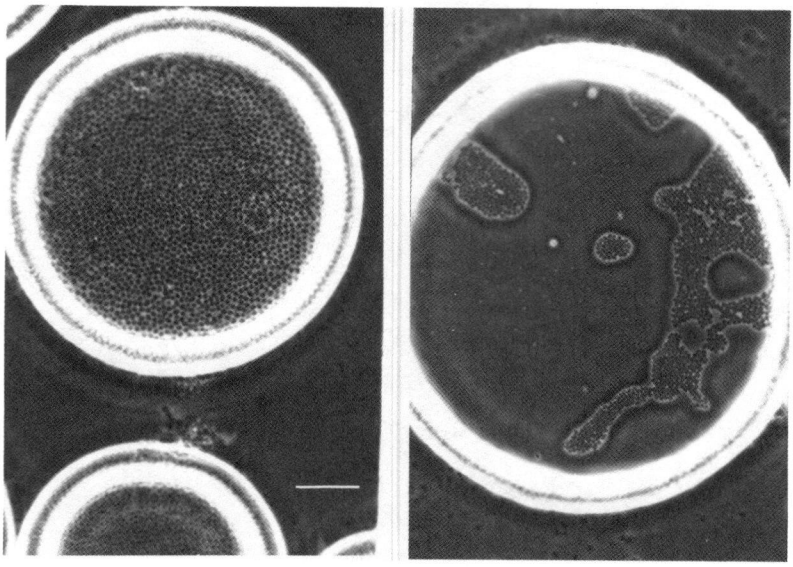


Figure 10-5 Bacteria-coated hexadecane droplets in the BATH test: a hydrophobic strain, *Serratia marcescens* RZ (*left*) and a hydrophilic one, *Serratia marcescens* 3164 (*right*). The bar denotes 10 μm.

the cell surface hydrophobicity and can be determined easily from the decrease in light absorbance of the lower aqueous phase, as compared to the absorbance of a control suspension. As an alternative to optical density measurements, bioluminescence has been used (Vanhaecke and Pijck, 1988).

The test tubes employed must be uniform and clean, preferably acid washed and all possess the same diameter. For these reasons Sharon *et al.* (1986) recommended that polystyrene cuvettes should be used in the BATH test. However, care must be taken, since hydrocarbon droplets can attach to the cuvette wall (H. C. Van der Mei, unpublished).

To allow optimal bacterial adhesion to the hydrocarbon droplets it is important to mix the suspension long enough. It is recommended that this be verified by varying the time of mixing when first applying the test.

Many investigators have modified the original BATH test with respect to the ionic strength, composition, and pH of the buffer (Nesbitt *et al.*, 1982; Rosenberg, 1984; Kozlovsky *et al.*, 1987; Mozes and Rouxhet, 1987). These seemingly small variations can greatly alter the results. When the ionic strength is increased by choosing another type of buffer or addition of salts the hydrophobicity of the bacteria is enhanced due to the elimination of the influence of electrostatic interactions. Especially for intermediate or weakly hydrophobic strains, addition of salts to the buffer is a good method to determine the rank order of the hydrophobicity of the strains.

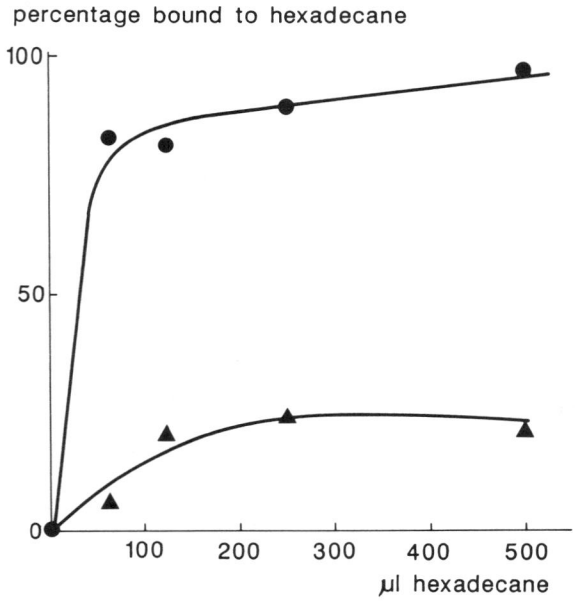


Figure 10-6 The percentage of *Serratia marcescens* RZ (●) and *Serratia marcescens* 3164 (▲) as a function of the hexadecane volume obtained in the original BATH test. The volume of the aqueous phase used was 3 ml.

Furthermore, different types of hydrocarbons can be used: *n*-hexadecane (Rosenberg *et al.*, 1980; Weiss *et al.*, 1982; Gibbons *et al.*, 1983; Ofek *et al.*, 1983; Knox *et al.*, 1985; Van Loosdrecht *et al.*, 1987; Van der Mei *et al.*, 1987a,b), *n*-octane (Rosenberg *et al.*, 1980; Kozlovsky *et al.*, 1987; Shinjo *et al.*, 1987), *p*-xylene (Rosenberg *et al.*, 1980; Hogt *et al.*, 1982; Sweet *et al.*, 1987), and toluene (Nesbitt *et al.*, 1982; Mozes and Rouxhet, 1987). Some investigators used and compared different hydrocarbons (Rosenberg *et al.*, 1980; Dillon *et al.*, 1986; Minagi *et al.*, 1986; Vanhaecke and Pijck, 1988). Most times, however, aliphatic hydrocarbons, e.g., hexadecane, appear to be the preferable hydrocarbon for the BATH test, since aromatic hydrocarbons may cause lysis of the bacterial cells (Vanhaecke and Pijck, 1988). It is also important to check both the lower aqueous phase and the upper "cream" hydrocarbon phase microscopically to ensure that the cells are indeed at the hydrocarbon–water interface (Fig. 10-5) and that the decrease in turbidity is due to adhesion, rather than to clumping or lysis.

10.3.2. Kinetic BATH Test

The original BATH test as described above has been criticized for an insufficient quantitative control of the experimental parameters. This stimulated Lichtenberg and co-workers to propose a more quantitatively controlled version of the BATH test, called the kinetic BATH test (Lichtenberg *et al.*, 1985).

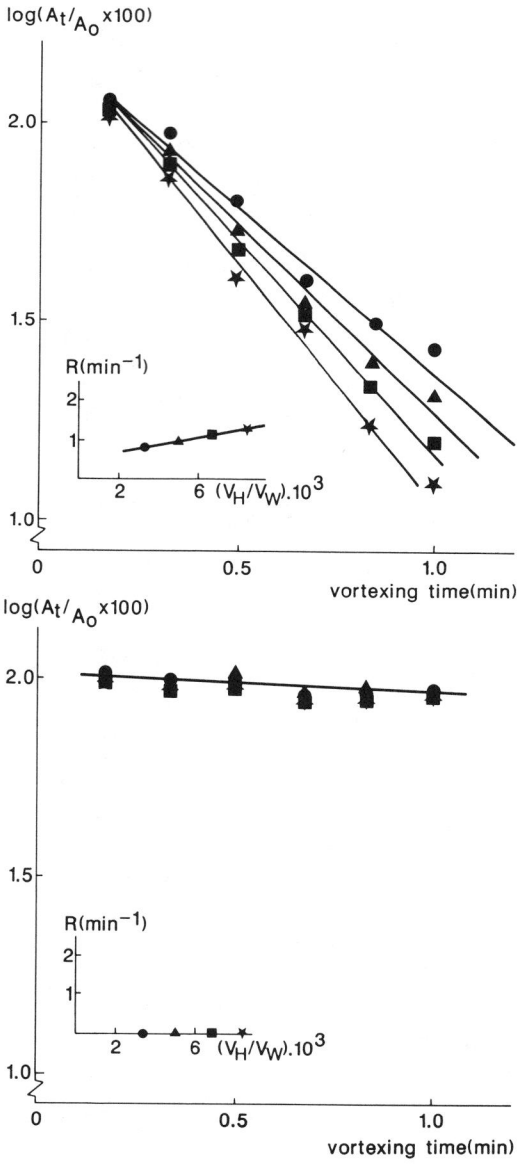


Figure 10-7 The optical density ratios, $\log(A_t/A_0 \times 100)$, of *Serratia marcescens* RZ (*top*) and *Serratia marcescens* 3164 (*bottom*) as a function of the vortexing time, for various hydrocarbon–water volume ratios V_H/V_W (★, ■, ▲, and ●). A_t is the optical density after vortexing, whereas A_0 is the optical density of a vortexed bacterial suspension (with no hexadecane added). The insert represents the slopes R of the various lines as a function of V_H/V_W.

The difference between the two tests can best be illustrated by an example. Figure 10-6 shows the percentage of *Serratia marcescens* RZ and *Serratia marcescens* 3164 bound to hexadecane as a function of the volume of hexadecane added to the bacterial suspension in the originally proposed BATH test. Vortexing was done during 60 sec and the optical density was measured at 600 nm, 15 min afterward. A clear difference between the hydrophobicity of the strains can be seen in addition to the importance of the hexadecane volume used. Figure 10-7 shows

the influence of vortexing time on optical densities obtained for these two strains with various hexadecane concentrations. In the kinetic BATH test the adhesion rates, i.e., the slopes of the lines, R (min^{-1}) in Figure 10-7 are plotted as a function of hexadecane–water volume ratio. The slope of this line, the so-called removal coefficient (Figure 10-7), may be considered a measure for the cell surface hydrophobicity. Hydrophobic strains in the original BATH test as e.g., *Serratia marcescens* RZ, will have a high removal coefficient in the kinetic test. Our experiments yielded a removal coefficient of 86 min^{-1} for *Serratia marcescens* RZ, contrary to almost zero for the hydrophilic mutant 3164.

One interesting anomaly of this approach is that back extrapolation to the y intercept (i.e., hydrocarbon volume approaching zero) usually yields positive values. This has been interpreted as an indication of the cooperative nature of adhesion at the oil–water interface (Sharon *et al.*, 1986).

10.4. HYDROPHOBIC PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS

Partitioning of bacteria over two immiscible aqueous liquid phases containing dextran and polyethylene glycol (PEG) can be used to investigate the hydrophilic or hydrophobic character of bacteria. In such a two-phase system hydrophobic bacteria partition preferentially into the PEG (top) phase, whereas hydrophilic bacteria accumulate in the dextran (bottom) phase. Sometimes, however, bacteria have a tendency to collect at the interface region.

The partitioning in aqueous two-phase systems as originally described by Albertsson (1970) can be summarized as follows: A small volume of a concentrated bacterial suspension is added to the dextran and PEG containing two-phase system. Subsequently, the phases are mixed thoroughly and allowed to separate. Sometimes partitioning of the bacteria over the two phases can be disturbed by sedimentation, particularly when separation takes longer than approximately 1 hr. From the bottom phase or the top and bottom phases 100 µl is withdrawn for the determination of the number of bacteria in the respective phases, as can be done for example by optical or radioactive methods (Miörner *et al.*, 1982).

The most crucial point of this method is the preparation of a good phase separation system. The separation of the two phases, dextran and PEG, is dependent on the temperature, concentration of the polymers, batch and molecular weight of the polymers, and salt concentration. The selection of a suitable phase system has to be based on the empirical variation of these different parameters and must be done prior to the actual experimenting with microorganisms. To increase the selectivity of the polymer two-phase system, hydrophobic groups have been covalently bound to the phase polymers (Eriksson *et al.*, 1976). Different fatty acids have been esterified with PEG such as palmitate-PEG and stearate-PEG in order to enhance partitioning. Fatty esters with different chain lengths or different

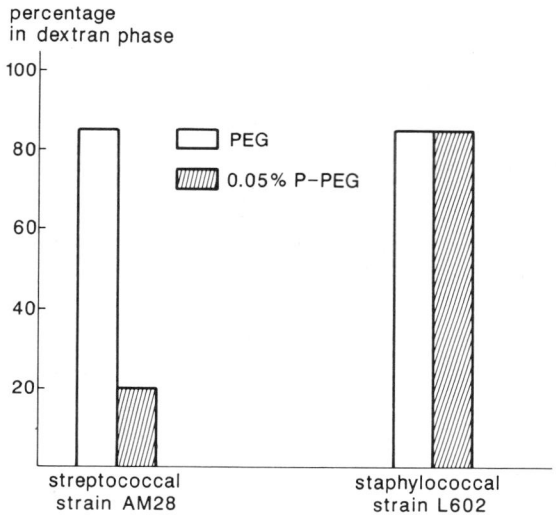


Figure 10-8 A comparison of the percentages of bacteria in the dextran phase with an unsubstituted (PEG) and a palmitate substituted polyethyleneglycol phase (P-PEG) for two different bacteria (adapted from Miörner *et al.*, 1982). Data are expressed as a percentage of the total number of cells added to the two-phase system.

numbers of double bonds have different effects on the partitioning (Miörner *et al.*, 1982). In this case the hydrophobic bacteria will go to the interface or top (PEG) phase, as illustrated in Figure 10-8.

10.5. HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Hydrophobic interaction chromatography (HIC) measures the adhesion of cells to columns of Sepharose beads that have been made hydrophobic (Smyth *et al.*, 1978). The beads are rendered hydrophobic by covalent binding of octyl- or phenyl residues.

Cells suspended in a buffer are applied to the columns and subsequently the percentage of cells eluted from the columns by various agents is determined by cell concentration measurements. Data can be expressed as a hydrophobic index (HI) defined as

$$HI = \frac{(\%\ \text{control eluted} - \%\ \text{octyl eluted})}{(\%\ \text{control eluted})} \qquad (10\text{-}6)$$

where the "control eluted" is the percentage of cells eluted from the unsubstituted Sepharose CL4B. According to Eq. (10-6), a hydrophobic strain yields a high

(close to 1) HI. The density of bacterial suspensions can also be determined radioactively (Hermansson *et al.*, 1982; Stenström, 1989).

HIC can be easily performed in small columns, prepared from Pasteur pipettes containing octyl- or pheynl-Sepharose-CL4B opposed to unsubstituted Sepharose-CL4B, which serves as a nonhydrophobic control (Clark *et al.*, 1985). Since bacteria may become bound to or entrapped by the Sepharose beads themselves (Olsson and Westergren, 1982; Clark *et al.*, 1985) it is of great importance to test the hydrophobized beads alongside the unsubstituted control.

To exclude the possibility that binding to the amphiphilic sepharose gels is due to mechanical trapping of bacterial aggregates induced by high salt concentrations (as happens sometimes in the standard HIC column procedure described above), the use of a batch procedure has been proposed (Jonsson and Wadström, 1983; Ascencio-Valle *et al.*, 1988). In the batch procedure the bacterial suspension is mixed with one of the Sepharose gels. To ensure complete mixing, the samples are gently agitated for 1 hr at room temperature. Thereafter the gel is allowed to settle and the visible appearance of the supernatant is recorded.

It is somewhat difficult to determine on the basis of a comparison of literature data whether a given strain is hydrophobic or hydrophilic on the basis of column chromatography because the results are very much dependent on the type and concentration of the salt (Jonsson and Wadström, 1983), temperature, nature of the hydrophobic ligand and its density on the matrix, and the pH of the eluent (Mozes and Rouxhet, 1987). In Figure 10-9, examples of possible effects of different types of salt and pH of the eluent are demonstrated.

10.6. SALT AGGREGATION TEST

The so-called salt aggregation test (SAT) is a simple technique by which one can infer the hydrophobicity of bacterial cell surfaces based on salt induced aggregation (Lindahl *et al.*, 1981). Hydrophobic bacteria tend to aggregate at lower salt concentrations than do hydrophilic strains due to the interplay of electrostatic and van der Waals forces between cells. At higher salt concentrations, the influence of electrostatic stabilization disappears and van der Waals interaction between bacteria causes aggregation.

The salt induced aggregation test as described by Lindahl *et al.* (1981) can be summarized as follows. A set of dilutions of ammonium sulfate is made in sodium phosphate buffer pH 6.8 (0.002 *M*) over the range of 4.0–0 *M*. A small volume of a bacterial suspension positioned on a microscope glass slide is then mixed with an equal volume of salt solution during 2 min at room temperature. Subsequently the minimal salt concentration (SAT) yielding visible aggregation is determined by comparing the aggregation in a salt-phosphate solution with the aggregation in phosphate buffer (negative control).

Visual observation of bacterial aggregation is very dependent on the density of the bacterial suspension and it is advisable that a range of cell densities should

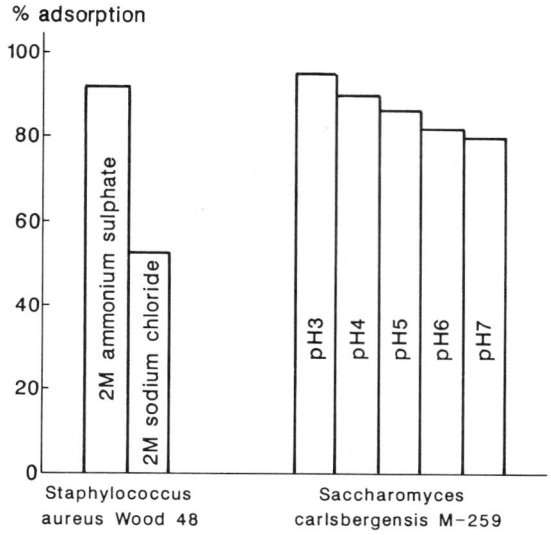


Figure 10-9 The percentage adsorption of *Staphylococcus aureus* Wood 46 (Jonsson and Wadström, 1983) to octyl-sepharose in hydrophobic interaction chromatography employing different salts and the influence of pH on the percentage adsorption of *Saccharomyces carlsbergensis* M-259 (Mozes and Rouxhet, 1987). Note that, due to the lack of nonhydrophobic control data, the results are not expressed as hydrophobic indices according to Eq. (10-6).

be tested before the actual experiments. Other experimental variables included the type of ammonium salts, pH, temperature, and duration of mixing.

The salt concentration needed for bacterial aggregation can vary over wide ranges. Lindahl *et al.* (1981) for instance using a range of fimbriated and nonfimbriated *Escherichia coli* strains observed aggregation at as little as 0.05 *M* of ammonium sulfate, whereas Van der Mei *et al.* (1987b), working under identical conditions noted that final concentration of at least 1.0 *M* ammonium sulfate was required in order to induce aggregation of oral streptococci (Table 10-3).

Table 10-3 Relative Surface Hydrophobicities of *Escherichia coli* Strains (Lindahl *et al.*, 1981) and Streptococcal Strains (Van der Mei *et al.*, 1987b) Determined by the Salt Aggregation Test (SAT)

Strain	SAT (*M*)
E. coli 2683	≤ 0.01
E. coli D563	0.05
E. coli 4845	2.0
S. salivarius HB	1.0
S. mitis L1	2.5
S. sanguis CH3	>2.0

10.7. MISCELLANEOUS TECHNIQUES

Under most circumstances the techniques described will allow determination of "bacterial hydrophobicity." However, in some cases the limitations of the methods discussed have prompted investigators to propose alternative assays. Probably the number of less familiar methods is large and we pretend by no means to be complete in this summary of miscellaneous techniques.

Sar (1987), for example, was unable to apply the contact angle technique to his particular strains and modified the test by determining the direction of spreading of a droplet positioned at the border between a bacterial layer and agar, glass, or polystyrene. Adsorption of nonionic surfactants (Noda and Kanemasa, 1986) to bacterial cell surfaces has also been proposed to study the presence of hydrophobic sites on cell surfaces. Hazen and Hazen (1987) mixed polystyrene microspheres with a yeast suspension and determined subsequently the number of microspheres attached to the cell surface. Rosenberg (1981) studied bacterial adhesion on polystyrene replicas of bacterial colonies on agar, obtained by pressing a polystyrene disk onto the colonies on the agar surface. Since hydrophobic strains left imprints on the replica disks, whereas nonhydrophobic cells were washed off, the remaining cells serve as a measure for hydrophobicity.

The last two methods are completely adhesion based, as are BATH and HIC, and the outcome of each method is determined by all parameters involved in adhesion, including not only hydrophobicity but also the presence of surface appendages and localized groups. This is in contrast to tests aimed at measuring the overall hydrophobicity of a cell surface, as, for example, the contact angle technique does.

10.8. SELECTED EXAMPLES OF HYDROPHOBICITY MEASUREMENTS TO STUDY BACTERIAL CELL SURFACES

10.8.1. Encapsulated and Nonencapsulated Staphylococcal Strains

Coagulase-negative staphylococci have been recognized as important pathogens in hospitalized patients. Coagulase-negative staphylococcal strains are especially involved in infections associated with prosthetic implants and medical devices. The initial step in the pathogenesis is the adhesion of the bacteria to the artificial surfaces (Dankert *et al.*, 1986). The hydrophobic character of the bacterial cell surface plays an important role in the adhesion to substrata. Therefore several investigators have measured the hydrophobicity of coagulase-negative staphylococcal strains. Hogt *et al.* (1985) determined the hydrophobicity of the

cell surface of 7 encapsulated and 16 nonencapsulated coagulase-negative staphylococcal strains with the BATH test using xylene. Their encapsulated strains had a more hydrophobic character than the nonencapsulated strains, whereas large variability was observed among the different nonencapsulated strains. Van der Mei *et al.* (1989) observed no difference in the water contact angle, used as a hydrophobicity measure, between three encapsulated and three nonencapsulated coagulase-negative staphylococcal strains. However, on the same set of strains, a significantly lower formamide contact angle was detected on the encapsulated strains compared to the nonencapsulated ones, resulting in a zero hydrogen-donating surface free energy parameter and consequently, a zero acid–base surface free energy component γ_{bv}^{AB} in the approach of Van Oss *et al.* (1987) for the nonencapsulated strains. Thus contact angle measurements, followed if desired by surface free energy calculation according to the approach of Van Oss *et al.* (1987), might offer an alternative way for identifying the presence of a thin capsule that is otherwise difficult to detect by India ink staining. Similarly, adhesion to toluene was found to have the highest positive predictive value, when compared with other diagnostic techniques, for identifying pathogenic coagulase-negative staphylococcal isolates (Martin *et al.*, 1989).

Encapsulated *Staphylococcus aureus* strains isolated from bovine mastitis and from human urinary tract infections showed very high surface hydrophobicities determined by the salt-aggregation test (Mamo *et al.*, 1987) and hydrophobic interaction chromatography (Jonsson and Wadström, 1983). HIC revealed however that nonencapsulated variants were more hydrophobic and it was recommended that HIC could be used as a method for detecting hydrophilic carbohydrate capsules, microcapsules, or other carbohydrate surface structures.

10.8.2. Halobacteria

Halobacteria are found in places where high salt concentrations are available as, for example, in salterns and salt lakes like the Dead Sea. The cell surface characteristics of halobacteria are the results of their adaptation to the high salt concentration of the media. These adaptations have been shown to be highly specific mechanisms of survival and are useful only in a particular salt regime. The balance between charged and nonpolar groups on the cell surface in halobacteria may be important in determining its degree of halotolerance and/or halophilism (Ascencio-Valle and Ochoa, 1988). This property necessarily varies from one species to another and its evaluation may be an interesting approach for classification.

The HIC test is ideal for determining the hydrophobicity of halobacteria because of its salt-dependent fractionation procedure. Five bacterial strains with different ranges of halotolerance and/or halophilism showed distinct chromatographic patterns but no correlation between the hydrophobic character of the bacteria and its salt tolerance or halophilism was found (Ascensio-Valle and Ochoa, 1988). However, three extremely halophilic bacterial strains, *Halobacterium cutirubrum*, *H. halobium*, and *H. salinarium*, did show distinctly different

hydrophobicities among each other (Ochoa and Ascencio-Valle, 1988), suggesting that hydrophobicity tests can be used for cell classification purposes. In this respect, it is interesting to note that the above three strains, historically regarded as different species, are now classified under one species, namely *H. salinarium* (Krieg, 1984).

10.8.3. Screening of the Surface Properties of Isogenic Variants

It is known from the literature that the BATH test (Rosenberg and Rosenberg, 1981; Gibbons *et al.*, 1983) and also HIC can be used for the isolation of nonhydrophobic mutants from parent strains. Furthermore, it is also possible to use hydrophobicity tests for the discrimination of isogenic variants.

Van der Mei *et al.* (1987a) observed a large difference in hydrophobicity between the parent strain *S. salivarius* HB and a range of mutants with defined differences in surface structures. The hydrophobicity was determined with the kinetic BATH test, contact angle measurements, and HIC, and the results of all tests showed good relationships for these closely related strains (Van der Mei *et al.*, 1987b). The different hydrophobicity levels of the strains could be associated with the absence or presence of different classes of fibrils. The completely bald mutant *S. salivarius* HBC12 was the most hydrophilic one.

Busscher *et al.* (1991) also investigated a series of hydrophobic tufted *S. sanguis* strains and found that the bald mutant strain CR 311 VAR3 was the most hydrophilic, as determined with the BATH test as well as with contact angle measurements.

10.9. CONCLUDING REMARKS

The choice of a given method to study bacterial cell surface hydrophobicity will be inspired mostly by the experimental possibilities available. This makes it extremely important to realize that although all tests claim to measure "hydrophobicity," it is difficult to directly relate bacterial hydrophobicities derived in various ways (Dillon *et al.*, 1986; Mozes and Rouxhet, 1987; Van der Mei *et al.*, 1987b). Roughly speaking, the methods discussed can be divided in two groups:

1. methods that measure the overall macroscopic hydrophobic properties of the cell surfaces like, e.g., the contact angle technique, two-phase partitioning or salt induced aggregation; and
2. methods that probe bacterial adhesion to hydrocarbon droplets, hydrophobic Sepharose beads, or polystyrene can be considered indicative for the presence of hydrophobic groups at the cell surface. As this group of methods is adhesion based, the outcome of a test will reflect all parameters involved in microbial adhesion including, among others, Van der Waals forces and electrostatic interactions.

Table 10-4 Linear, r_l, and Rank, r_s, Correlation Coefficients between Four Different Hydrophobicity Tests, as Employed on 29 Different Oral Streptococcal Strains[a]

r_s \ r_l	θ_{H_2O}	BATH	HIC	SAT
θ_{H_2O}[b]	–	0.27	0.09	−0.01
BATH	0.31	–	0.04	0.35
HIC	−0.12	0.04	–	−0.37
SAT	−0.05	0.39	0.27	–

[a]Adapted from Van der Mei *et al.* (1987b).
[b]Water contact angle on bacterial deposits on membrane filters.

It should be noted that despite this rough division, even within one group of seemingly similar tests, one may obtain completely different results, also on a comparative level. This is illustrated in Table 10-4, in which the relationships between results of four different tests, applied on a wide variety of oral streptococcal strains are compared by a linear and a rank correlation test (Van der Mei *et al.*, 1987b). A total lack of correlation is obvious from the low correlation coefficients, in agreement with findings by Dillon *et al.* (1986) and Mozes and Rouxhet (1987), comparing a similar set of techniques on different collections of microorganisms. However, good correlations among different hydrophobicity tests were reported, when comparing extremely hydrophobic and hydrophilic microorganisms (Dillon *et al.*, 1986; Mozes and Rouxhet, 1987). Also Van der Mei *et al.* (1987a,b) observed good correlations when comparing a parent strain (*S. salivarius* HB) and its well-defined mutant strains, which differed only in the absence or presence of specific surface structures. More research is therefore required in order to unravel the mechanisms by which these techniques function and to raise them above the empirical level. We conclude that the hydrophobicity tests described all have some proven value with regard to bacterial adhesion, but that bacterial hydrophobicity values should never be given without carefully describing the method used.

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N. Mozes, P.S. Handley, H.J. Busscher and P.G. Rouxhet

This unique text is a critical description of up-to-date methods used by microbiologists and physical chemists to study microbial cell surfaces. Microbial Cell Surface Analysis includes information on adhesion and cell surface architecture, electron microscope techniques for cell surface analysis, physico-chemical methods for the detection of cell surface chemical composition, hydrophobicity and electrical charge properties and includes examples of studies using both structural and physico-chemical methods. It is aimed at microbiologists, immunologists, physical chemists, engineers and anyone who has an interest in bacterial surfaces, their properties, and how to study them.

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